0273-2289/87/1613—0201$02.00

A Conjugate of Trypsin and Chymotrypsin

Y. S. RAJPUT[1] AND M. N. GUPTA[2,*]

[1]*Animal Biochemistry Division, National Dairy Research Institute, Karnal–132 001; and* [2]*Chemistry Department, Indian Institute of Technology, New Delhi 110 016, India*

Received July 6, 1987; Accepted September 3, 1987

ABSTRACT

A heteroenzyme conjugate retaining activities of two component enzymes from trypsin and chymotrypsin was prepared using *N*-succinimidyl pyridyl dithiopropionate as crosslinking reagent. The conjugate bound to both trypsin and chymotrypsin affinity columns. Trypsin and chymotrypsin were linked in the ratio of 1:1 on mol basis. The conjugate, when treated with dimethyladipimidate, showed decreased autolysis of its trypsin component.

Index Entries: Heteroenzyme conjugate; crosslinking; trypsin autolysis.

INTRODUCTION

Protein-protein conjugates find a variety of applications in biochemistry (*1–3*). One such application has been the linking of two or three enzymes to create models for segments of metabolic pathways (*4*). Wang (*5*), on the other hand, linked DNase and RNase for the specific purpose of creating an efficient "killer" hybrid enzyme for destroying leukemia cells. We feel that such conjugates can also be useful in probing complex biological structures. To start with, we have attempted here to link together two well characterized proteolytic enzymes, viz., trypsin and chymotrypsin.

*Author to whom all correspondence and reprint requests should be addressed.

MATERIALS AND METHODS

Trypsin (3×crystalline, electrophoretically homogeneous) and chymotrypsin (electrophoretically homogeneous) were purchased from Sisco Research Laboratory, India. *N*-succinimidyl pyridyl-dithiopropionate (SPDP), dimethyladipimidate HC1 (DMA), and benzamidine-agarose were obtained from Sigma Chemical Co., USA. Sephadex G-25, sephadex, G-75, and sephadex G-100 were the products of Pharmacia Fine Chemicals, Sweden. 4-Phenyl butylamine-sepharose-4B was procured from Pierce Chemical Co., USA. Benzoyl DL-arginine *p*-nitroanilide (BAPNA) and benzoyl L-tyrosine ethyl ester (BTEE) were obtained from the Council of Scientific and Industrial Research Centre for Biochemicals, India. Bovine casein was prepared as described by Rajput and Ganguli (*6*). Coomassie brilliant blue G-250 was purchased from E. Merck, Darmstadt, Germany. Dithiothreitol (DTT) was a product of Fluka, Switzerland.

Preparation of the Trypsin-Chymotrypsin Conjugate

The multistep procedure was based on the one described by Carlsson et al. (*7*).

Introduction of 2-Pyridyl Disulphide Structure into Trypsin and Chymotrypsin

Five hundred nmol of trypsin or chymotrypsin was dissolved in 5 mL sodium barbitone (0.03 M) containing 0.3 M NaCl and 25 mM Ca^{++} (buffer A) pH 7.5. Five hundred μL of 200 mM SPDP (prepared in ethanol) was added slowly with constant stirring at 25°C. The reaction mixture was kept for 30 min at 25°C. The excess reagent was removed immediately by gel filtration on Sephadex G-25 column (1.3×55 cm) which was pre-equilibrated and eluted with buffer A, pH 7.5. Fractions in void volume were pooled and stored till further use at 5°C.

Thiolation of Trypsin

The trypsin-2 pyridyl disulfide derivative was dialyzed against acetate buffer (0.1 M, pH 4.5) containing 0.1 M NaCl and 25 mM Ca^{++} (overnight) at 5 °C and concentrated to 4 mL. The pH was again adjusted to 4.5 by adding acetic acid. The reduction was carried out by adding DTT (dissolved in a small volume of distilled water) to a final concentration of 25 mM. The reaction mixture was kept at 25°C for 30 min. The excess reagent was removed by gel filtration on a Sephadex G-25 column (1.3×55 cm), equilibrated, and eluted with buffer A (pH 7.5). Fractions in void volume were pooled and thiolated trypsin was used immediately for further reaction.

Conjugate Formation

Seven mL each of chymotrypsin-2 pyridyl disulfide derivative and thiolated trypsin (containing 400 nmol of each protein) in buffer A (pH

7.5) were mixed and kept at 25°C for 20 h. The conjugate formation takes place by a disulfide interchange reaction. The reaction mixture was dialyzed against water at 5°C and concentrated.

Estimation of 2-Pyridyl Disulfide Structure

The content of 2-pyridyl disulfide structure in modified enzymes was calculated by measuring the release of pyridine-2-thione after treatment with 50 mM DTT (30 min, 25°C), as described by Carlsson et al. (*7*). The concentration of trypsin, chymotrypsin, and pyridine-2-thione were calculated from their extinction coefficient values 1%/280 (trypsin) = 15.4 (*8*), 1%/280 (chymotrypsin) = 20.4 (*9*), and M/343 (pyridine-2-thione) = 8.08×10^3 (*10*).

Protein Assay

Protein was assayed by dye-binding method of Bradford (*11*) using Commassie brilliant blue G-250.

Enzymes Assay

Trypsin was assayed by the method of Erlanger et al. (*12*) using BAPNA as substrate. Chymotrypsin was assayed as described by Decker (*9*) with BTEE. For assaying activity of conjugate with casein, the method described by Rick (*13*) was employed.

RESULTS AND DISCUSSION

The main advantage of using SPDP as crosslinking reagent is that undesired trypsin-trypsin and chymotrypsin-chymotrypsin conjugates are not formed (*7*). This property of SPDP results in greater yield of the desired trypsin-chymotrypsin conjugate. For the trypsin-chymotrypsin conjugate preparation, both individual enzymes were reacted with SPDP and amino groups were modified. The extent of amino group modification can be calculated from the amount of pyridine-2-thione released after reducing the modified trypsin or chymotrypsin with DTT. The number of amino groups modified for chymotrypsin and trypsin were 0.6 and 1.05, respectively, per mol of enzyme. It was not possible to increase the extent of modification with either of enzyme because any further increase (beyond the reagent concentration given in methods) in SPDP concentration resulted in precipitation of the protein. Such a problem was also encountered by Carlsson et al. (*7*) in case of extensively modified proteins by SPDP. The probable reason given by these workers was the conformational changes caused by electrostatic effects associated with the conversion of the positively charged amino groups into neutral amide bonds. Carlsson et al. (*7*) also mentioned that under the experimental conditions, only a small fraction of the protein amino groups reacted with SPDP. The value

as low as 0.24 mol of pyridyl disulfide group introduced per mol of casein is also reported in the literature (*14*). On the other hand, 2.7 mol of pyridyl disulfide introduced in calmodulin has been used for preparation of cyclic nucleotide phosphodiesterase calmodulin conjugate (*15*).

The trypsin-chymotrypsin conjugate preparation was gel filtered on Sephadex G-100 column and formation of higher molecular weight species could be detected (Fig. 1). Fractions corresponding to elution volume 140–180 mL were pooled (called conjugate from now onwards) and studied further to confirm the formation of the conjugate. Since trypsin and chymotrypsin were joined together in the conjugate by the disulfide bond, the conjugate was treated with DTT and rechromatographed on the same

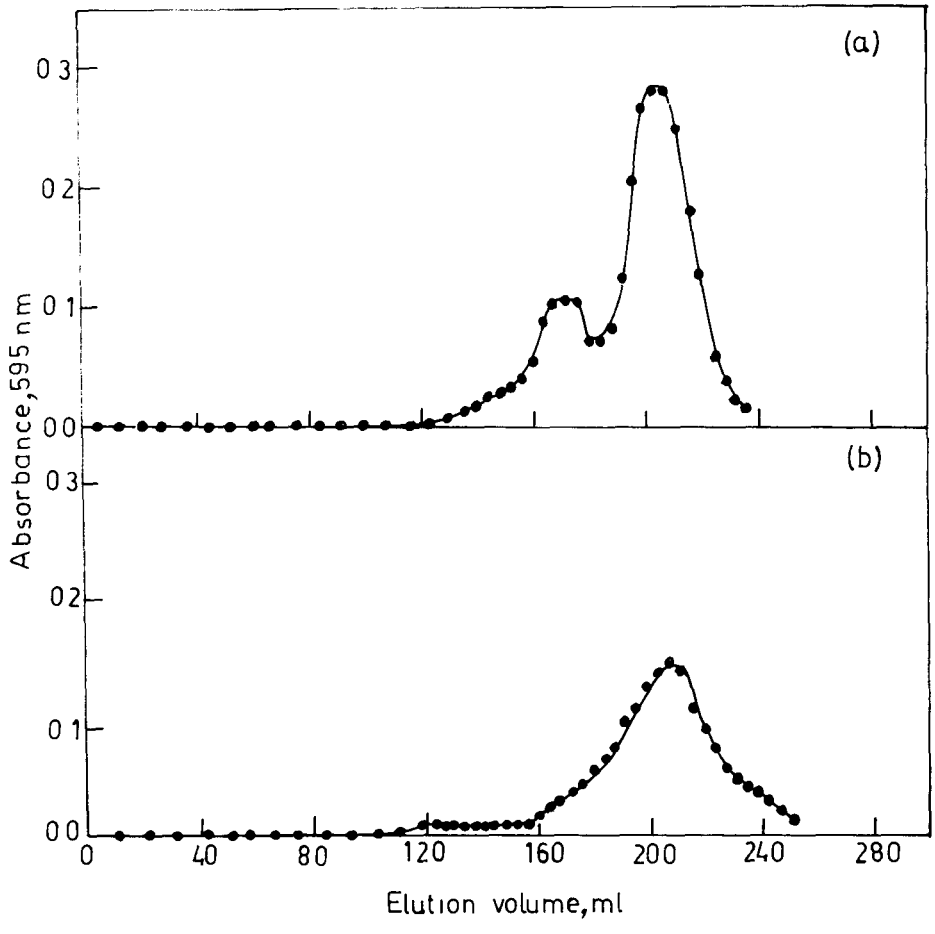

Fig. 1. Gel filtration of trypsin-chymotrypsin conjugate on sephadex G-100. Sephadex G-100 column (2×90 cm) was equilibrated and eluted with buffer A (pH 7.5) at 5°C. Flow rate and fraction size were 25 mL/h and 2 mL, respectively. Protein was estimated by Bradford method. (a) Profile of trypsin-chymotrypsin conjugate preparation. (b) Rechromatography of fractions corresponding to elution volume from 140 to 180 mL after DTT treatment (50 mM, 25°C, 30 min).

G-100 column. Since all the protein now eluted at the elution position of trypsin/chymotrypsin, this confirmed that the higher molecular weight material consisted of the two enzymes linked together by the disulfide bridge.

When the conjugate is passed through chymotrypsin affinity column, total chymotrypsin activity and most of the trypsin activity bound to the column (Fig. 2). This indicated again that the trypsin was covalently linked to the chymotrypsin. Slight trypsin activity observed unbound perhaps represented the unconjugated trypsin contaminent. The conjugate was also loaded on trypsin affinity column. In this case, the conjugate before loading was further purified by doing a regelfiltration on Sephadex G-100. All the trypsin and chymotrypsin activity was found to bind to the affinity column. The binding behavior of the conjugate to both affinity media further confirmed formation of the desired trypsin-chymotrypsin conjugate.

The conjugate formed was then tested for catalytic activities of individual enzymes. The conjugate had both tryptic and chymotryptic activities measured with BAPNA and BTEE as substrates, respectively. When specific activities of conjugate were compared with respective native enzymes, the conjugate was found to contain 28% tryptic and 54% chymotryptic activity. As the molecular weights of trypsin and chymotrypsin are same, this means that the trypsin component retained 56% activity and chymotrypsin component retained full activity. The loss to the extent of 27–34% in enolase activity was also observed when this enzyme was reacted with SPDP (4). These authors have also reported 37% loss in pyruvate kinase activity during conjugate formation when a heteroenzyme conjugate from enolase and pyruvate kinase was prepared. The trypsin-chymotrypsin conjugate was also tested for its activity towards large molecular weight substrate such as casein and the results are shown in Fig. 3. Since trypsin and chymotrypsin both hydrolyase casein, conjugate activity was compared with a mixture of trypsin and chymotrypsin mixed in the ratio of 1:1 on mol basis, and it was found that the proteolytic activity of the conjugate was marginally lower. However, when one considers that the trypsin component had lost substantial activity as a result of chemical modifications (involved during conjugation), there seems to be in fact a slight enhancement in caseinolytic activity purely because of conjugation *per se*.

The molecular weight of the trypsin-chymotrypsin conjugate was determined by gel filtration method on a calibrated Sephadex G-75 column. This was found to be 46,800 (Fig. 4). This value of the molecular weight suggested that trypsin and chymotrypsin were present in the ratio of 1:1 on mol basis in trypsin-chymotrypsin conjugate.

Since trypsin is known to undergo autodigestion at alkaline pH, trypsin-chymotrypsin conjugate was also tested for its stability *vis-a-vis* autolysis. The trypsin-chymotrypsin conjugate retained 52% activity toward BAPNA after 9 h incubation at 40°C, whereas under identical conditions, the mixture of trypsin and chymotrypsin retained 43% activity (Fig. 5).

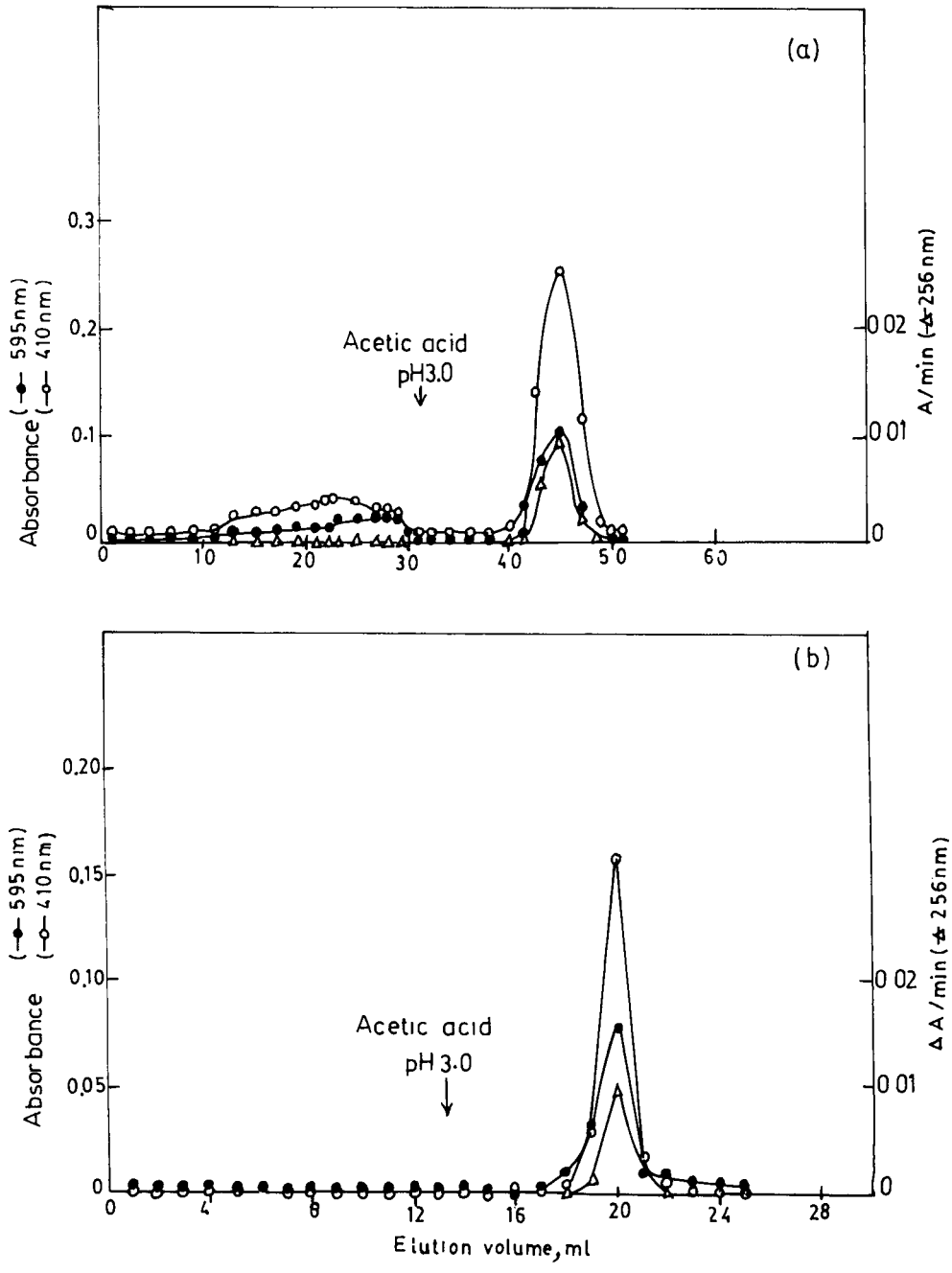

Fig. 2. Binding of trypsin-chymotrypsin conjugate on affinity columns. The conjugate was loaded on either (a) phenylbutylamine-sepharose 4B column (0.9×13 cm) or (b) benzamidine-agarose column (0.5×11 cm) equilibrated with buffer A (pH 7.5) and eluted with same buffer. At indicated point, the elution medium was changed to dil. acetic acid (pH 3.0). (●——●), protein by Bradford method; (○——○), trypsin activity; (△——△), chymotrypsin activity.

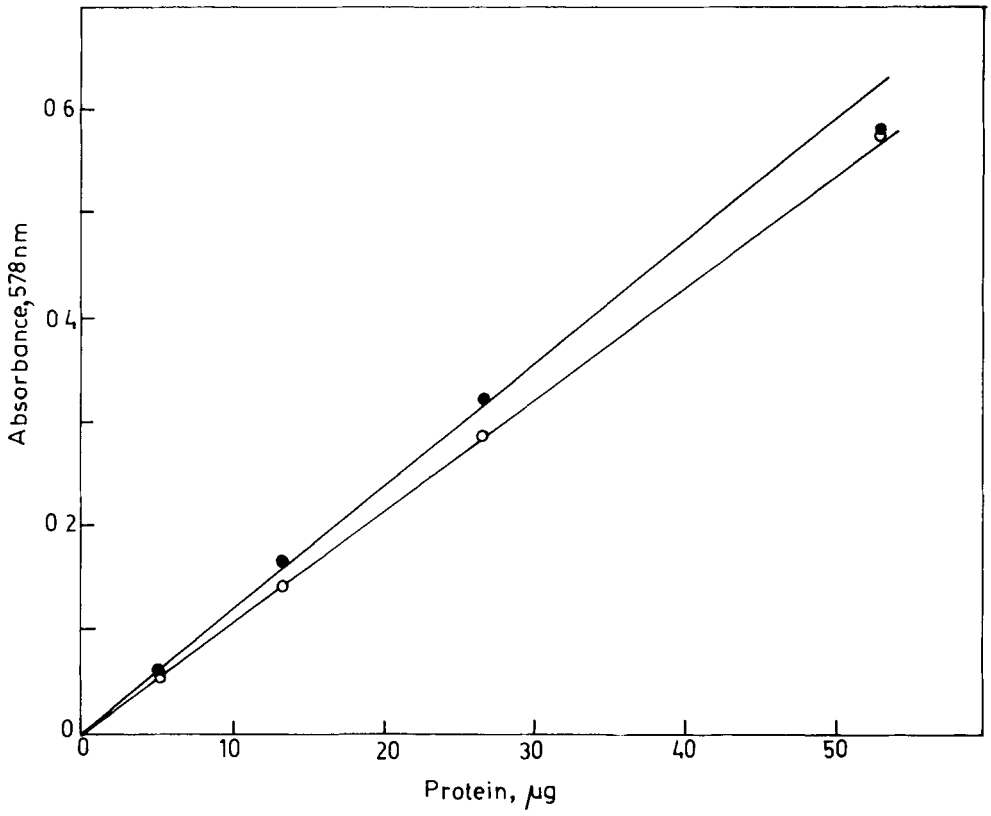

Fig. 3. Caseinolytic activity of native enzymes and trypsin-chymotrypsin conjugate. Caseinolytic activity was measured with bovine casein as the substrate. (○——○), trypsin-chymotrypsin conjugate; (●——●), trypsin and chymotrypsin when mixed in 1:1 ratio on mol basis.

These data showed that there was only marginal improvement in stability. As native trypsin has been successfully stabilized by bisimidoesters (*16*) the trypsin-chymotrypsin conjugate was reacted with this bisimidoester and autolysis was reinvestigated. The autolysis pattern of the DMA-treated conjugate is shown in Fig. 6. DMA-treated conjugate showed remarkable stability as compared to DMA-untreated conjugate. However, owing to DMA treatment, the trypsin-chymotrypsin conjugate lost 45% of its tryptic activity.

The trypsin-chymotrypsin conjugate was also studied for its storage stability in buffer A (pH 7.5). Trypsin and chymotrypsin activity of the conjugate were 95 and 100%, respectively, after 13 d storage at 5°C. The chymotrypsin activity was reduced to 50% when stored at −5°C for 15 d and subjected to freezing and thawing cycles four times during this period.

To conclude, we have intentionally chosen a difficult system (e.g., one of the components of trypsin having the properties of autolysis) and explored the preparation and properties of a hybrid enzyme. It is hoped

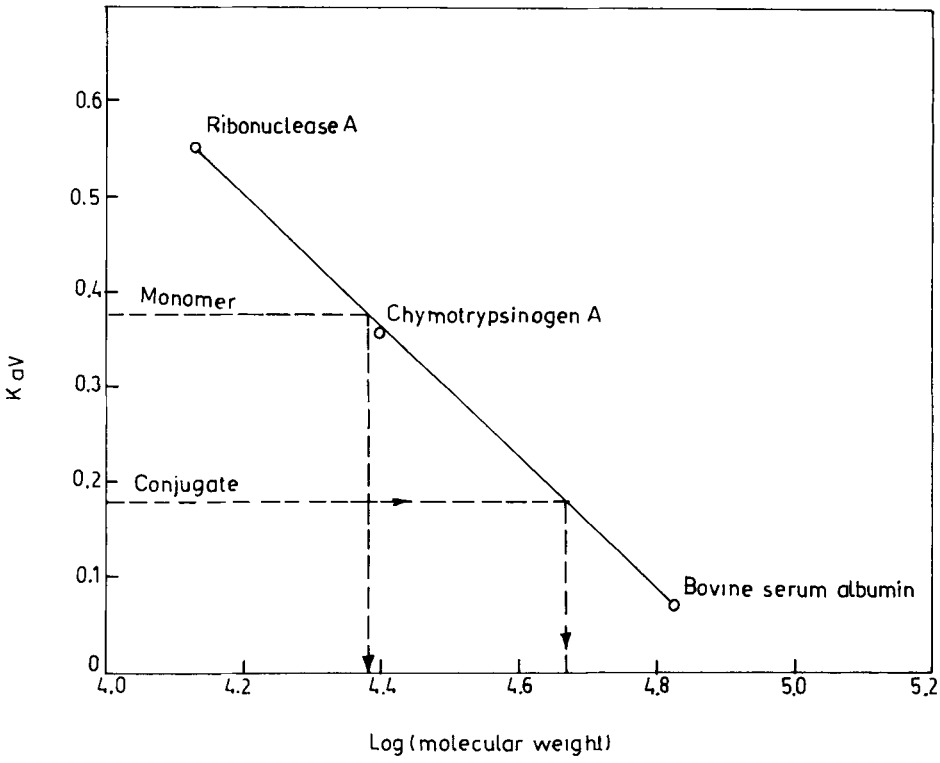

Fig. 4. Plot of Kev vs log molecular weight. Kav values for standard proteins were obtained from a column of Sephadex G-75 (3.3×60 cm) which was equilibrated with buffer A, pH 7.5 at 5°C. Molecular weight of trypsin-chymotrypsin conjugate and monomer proteins were calculated from standard curve.

that such hybrid enzymes would turn out to be useful novel reagants for determination of the structure of complex biological structures.

REFERENCES

1. Shier, W. T. (1985), *Meth. Enzymol.* **112,** 248-258.
2. Voller, A., Bidwell, D. M., and Bartlett, A. (1976), *Bull. World Health Organ.* **53,** 55-65.
3. Yoshitake, S., Yamada, Y., Ishikawa, E., and Masseyeff, R. (1979), *Eur. J. Biochem.* **101,** 395-399.
4. Ikura, K., Okumura, K., Sasaki, R., and Chiba, H. (1984), *Agric. Biol. Chem.* **48,** 355-364.
5. Wang, D. (1979), *Biochem.* **18,** 4449-4452.
6. Rajput, Y. S. and Ganguli, N. C. (1982), *Asian J. Dairy Res.* **1,** 13-16.
7. Carlsson, J., Drevin, H., and Axen, R. (1978), *Biochem. J.* **173,** 723-737.
8. Decker, L. A. (ed.) (1977a), *Worthington enzyme manual,* 221-224, Worthington Biochemical Corp., Freehold, NJ, USA.

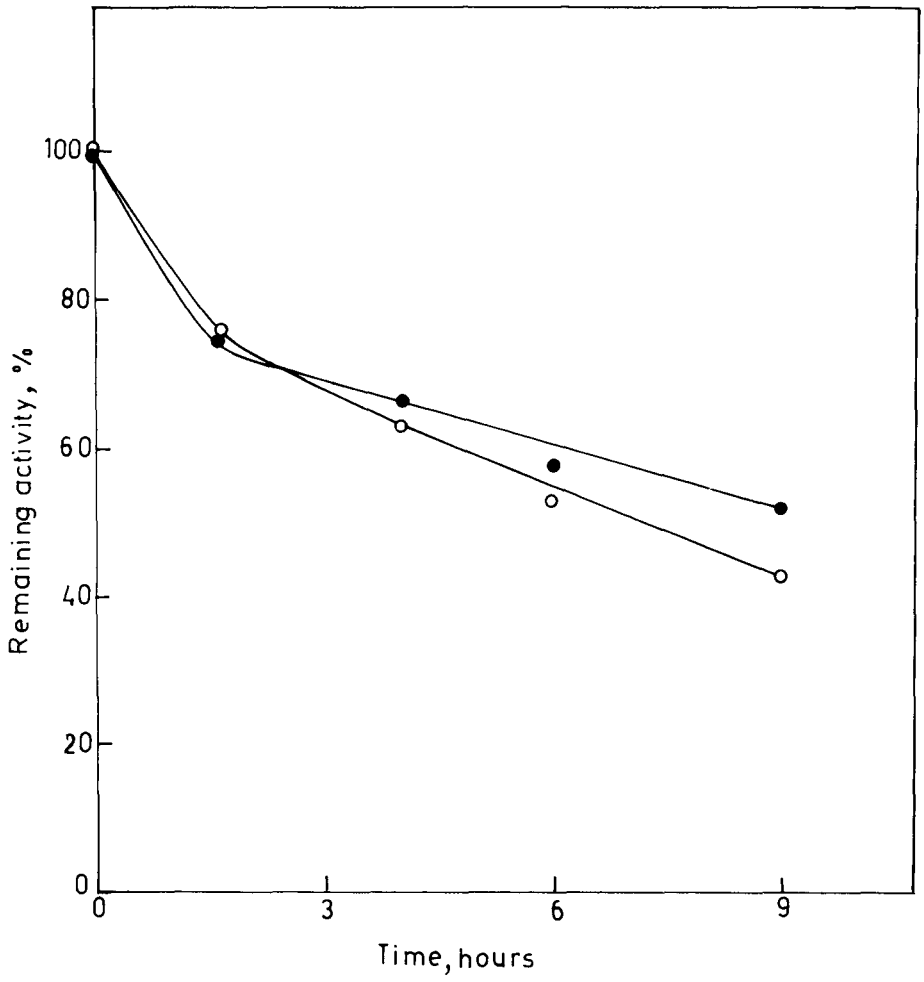

Fig. 5. Autolysis of trypsin-chymotrypsin conjugate. Autolysis was studied in buffer A (pH 8.3) containing 50 mM EDTA at 40°C and at a protein concentration of 100 μg/mL. EDTA (pH 8.3) was added to enable to study autolysis in the absence of Ca^{++}. Remaining tryptic activity was measured with BAPNA. For assaying tryptic activity, assay buffer contained 50 mM Ca^{++} instead of normally used 20 mM Ca^{++} so as to take care of the EDTA present in the autolysis system. Autolysis of a mixture of trypsin and chymotrypsin was also studied under identical conditions for comparison. (●——●), trypsin-chymotrypsin conjugate; (○——○), a mixture of trypsin and chymotrypsin mixed in the ratio of 1:1 on mol basis.

9. Decker, L. A. (ed.) (1977b), *Worthington enzyme manual,* 215-220, Worthington Biochemical Corp., Freehold, NJ, USA.
10. Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., Brocklehurst, K., Herbert, J. A. L., and Suschitzky, H. (1975), *Biochem. J.* **151,** 417-432.
11. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248-254.
12. Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys.* **95,** 271-278.

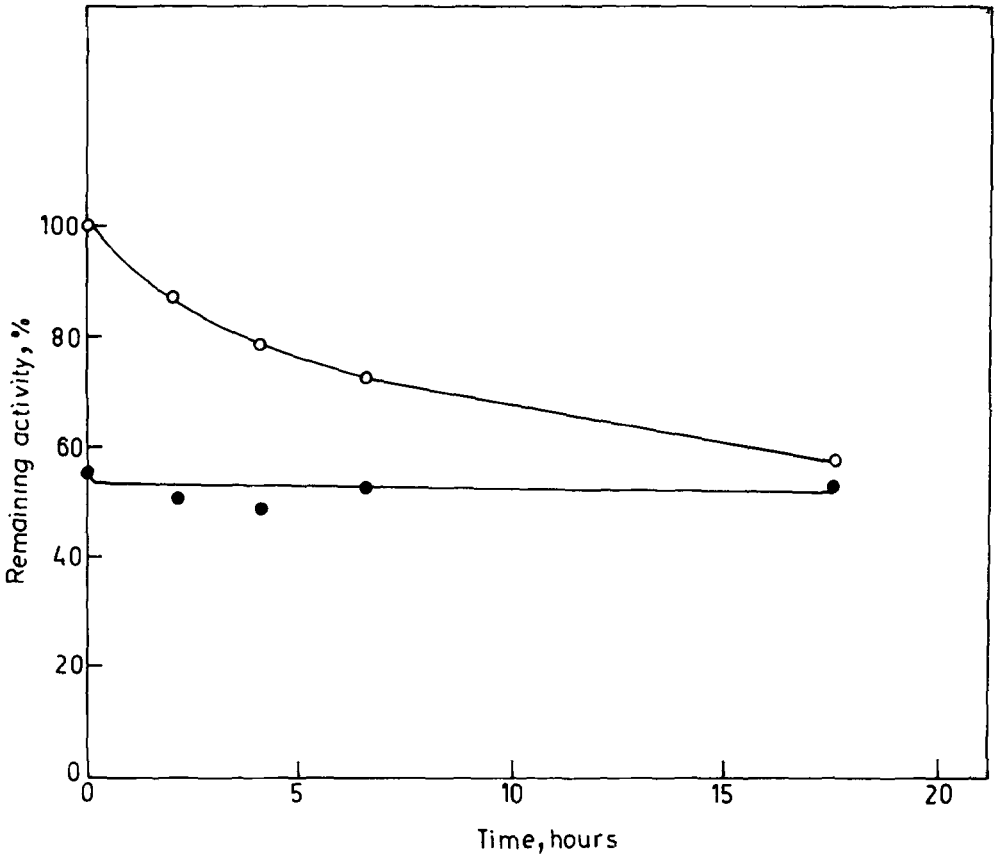

Fig. 6. Stabilization of trypsin-chymotrypsin conjugate by DMA. The conjugate (72 μg) was mixed with 15 mg DMA dissolved in buffer A (pH 8.3) and kept for 1 h at 25°C. Autolysis was studied as described in Fig. 5 except at a protein concentration of 44 μg/mL. For comparison, autolysis of unreacted trypsin-chymotrypsin conjugate was also studied under identical conditions. Remaining tryptic activity of DMA untreated (○——○) and DMA treated (●——●) conjugate expressed as percent of initial activity of untreated conjugate.

13. Rick, W. (1974), in *Methods of Enzymatic Analysis* (Bergmeyer, U. ed.) vol. 2, 2nd ed., pp. 1013–1024, Verlag Chemie Gmb-H., Weinheim.
14. Okumura, K., Ikura, K., Yoshikawa, M., Sasaki, R., and Chiba, H. (1978), *Agric. Biol. Chem.* **48,** 2435–2440.
15. Kincaid, R. L. (1984), *Biochem.* **23,** 1143–1147.
16. Rajput, Y. S. and Gupta, M. N. (1987), *Enz. Microb. Technol.* **9,** 161–163.